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This report is based on two meetings held in Europe in December 1989, and April 1990, as well as Dr. Cooksey's observations. Specifically, the meetings were: (a) "Physiology of Immobilized Cells" held in Wageningen, the Netherlands, and organized by the European Federation of Biotechnology and the Agricultural University at Wageningen; (b) "Immobilized Cell Processes" organized by the Society for General Microbiology of the United Kingdom at Warwick University. Proceedings of the first conference will be available in late 1990 from Elsevier. The second conference will not be published. The general interest in biotechnological production systems using fixed-cell reactors is increasing. Two similar meetings being held in less than 6 months is a strong indication of the interest. Although these meetings dealt specifically with the biotechnological side of cellular immobilization, there are aspects of this research that have importance in other fields.

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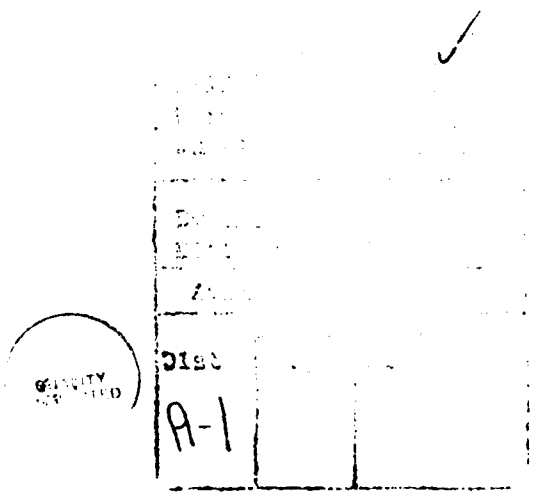

Immobilized Cell Research

Preface

The following is based on two meetings held in Europe in December 1989, and April 1990, as well as my own observations. Specifically the meetings were: (a) "Physiology of Immobilized Cells" held in Wageningen, the Netherlands, and organized by the European Federation of Biotechnology and the Agricultural University at Wageningen; (b) "Immobilized Cell Processes" organized by the Society for General Microbiology of the United Kingdom at Warwick University. Proceedings of the first conference will be available in late 1990 from Elsevier. The second conference will not be published.

Introduction

The general interest in biotechnological production systems using fixed-cell reactors is increasing. Two similar meetings being held in less than 6 months is a strong indication of the interest. Although these meetings dealt specifically with the biotechnological side of cellular immobilization, there are aspects of this research that have importance in other fields. Such systems have been proposed as simple mimics of natural biofilms. All the physical and chemical analytical problems associated with research on biofilms of natural origin are also seen in systems where cells have been immobilized intentionally for biotechnological reasons. Thus, cells immobilized in films can be used as models for processes in biofilms. They offer the possibility of investigating cell-cell interactions and other consortial processes in a defined system - investigations that are not really possible in natural films.

There are several perceived advantages to using fixed biocatalysts in production systems, the most important of which is touted as economy of operation. However, from the papers presented, it seems there is not yet universal agreement about the scientific reasoning behind this statement. Nevertheless, the overall impression I gained was that immobilized cells are genetically more stable, are protected from hydraulic shear stresses, and higher densities of biocatalysts (cells) can be achieved. As a result, this leads to shorter contact times in the reactor. When reporting on conferences such as these, generalizing can create problems. Causes can be the diversity of techniques used to immobilize cells, the extremely different types of organisms, and the many techniques used to compare cellular physiologies. Undoubtedly, any technique used to define the physiology of an immobilized cell must not change the environment in which the cell is living. The same, of course, applies to biofilm research. Intuitively obvious? Unfortunately not, and many investigations in the past have used homogenization of the fixed film of cells as a preliminary step to making measurements. The reasons for this are clear; it is difficult to sample reproducibly a film of cells or cells imbedded in a matrix. The conferences therefore stressed noninvasive techniques in assessing immobilized cell physiology. These techniques are just as relevant to the study of natural biofilms as they are to the systems described here.

Significance to the Navy of the Research Described

The research described below is of direct importance to those who guide the Navy's activities in biological fouling, biocatalysis, bioremediation, microbially enhanced corrosion, biotechnology, and molecular biology in general. Scientists involved in microanalysis of biological phenomena will also be interested.

Deterioration of man-made structures in the marine environment through the agency of microorganisms is possibly the largest single biological deterioration problem facing the Navy. The techniques reviewed here should be of particular use in biofouling studies, especially those involved in corrosion. As an example, I cite the ideas to use mixed microbial species purposely immobilized in an inert matrix containing colloidal iron particles, together with the various metabolic monitoring schemes. Similarly, the preservation of microbial activity in immobilized biocatalytic reactors is important in any biotechnological endeavor. We know little, for instance, of the behavior of Archebacteria in fixed films (except methanogens, of course).

The most immediately practical aspect of the research described is in the application of microbiology to remediate pollution. Immobilized cell or fixed-film reactors, especially if they are small and can be run continuously, are ideal for shipboard use. None, so far as I am aware, are in current use although massive systems are the norm in land-based sewage treatment plants. Pollutants, apart from being environmentally destructive, are also strategically important as tracers. There are therefore several reasons why a ship at sea should not leave evidence of its presence. The systems and means for their monitoring described herein should be useful in assessing the current state of research in this area, as well as suggesting direction for programs aimed at measuring microscale biological phenomena.

Why Use Immobilized Cells in Biotechnology

Performance. In general, there appeared to be no compelling biological reasons for adopting an immobilized cell system to carry out a biological transformation

over a process based on suspended cells. Immobilization of cells in a matrix is an additional step in the biotechnological process. Moreover, in most cases this step has to be carried out aseptically. Thus, immobilized cell processes are only potentially useful if they offer some considerable procedural advantage, such as in the recovery of product; i.e., downstream processing. In each case, therefore, the procedural advantages must be weighed against the investments to obtain them.

In a process where immobilized cells produce material that must be removed from the growth medium, it is obvious that a reactor effluent stream free of cells enables an easier, and hopefully cheaper, product recovery. There are instances where cells immobilized in a continuous-flow reactor can be a further procedural advantage over suspended cells. Where there is considerable preparation time before cells begin forming the required product, a system where the biological process is poised at the production stage is obviously advantageous.

Michael Dempsey, Manchester Polytechnic Institute, England, made this point rather clearly using as an example *Zymomonas mobilis* immobilized naturally on coke; i.e., no matrix was used to keep the cells in place. In this system, run as a fluidized bed reactor, Dempsey's group achieved flow rated equivalent to a 11.5 times μ_{max} with a biomass concentration 15 times that for suspension cultures in conventional stirred reactors. Even so, the concentration of continuously prepared ethanol in the effluent medium was only 7 percent (cf batch fermented ethanol about 12 percent). As was pointed out by a questioner (Nicholas Emery, University of Birmingham, England), a continuous process such as this one is unlikely to be very cost effective unless all the substrate (glucose in this case) is used up on a single pass through the fluidized bed. In those experiments, 90 percent of the glucose was used.

Ethanol is a primary cellular metabolite; i.e., one that is produced as a result of growth. Since growth in cells that are immobilized is somewhat restricted, it is more reasonable to consider secondary metabolites for production by such systems. In this context, B. Hahn-Hägerdal, University of Lund, Sweden, showed that the production of penicillin G was slower in immobilized *Penicillium chrysogenum* than in washed freely suspended mycelium. In contrast, there was a 1,000-fold increase in capsaicin production in immobilized cultures of a plant cell line from *Capsicum frutescens* (V. Bringi and M.L. Shaler, Cornell University, Ithaca, New York). Similarly, alkaloids are retained at an enhanced level in the medium by the ergot fungus *Claviceps purpurea* when it is entrapped in a calcium alginate gel compared to the situation when the cells are freely suspended. Production of alkaloids was, however, slower in entrapped cells (M. Lohmeyer et al., University of Münster, Federal Republic of Germany [FRG]).

Plasmid Stability. The loss of plasmid recombinant-DNA from commercially used genetically engineered cells is a problem inherent in its nongenomic location. There appears to be general agreement that immobilized plasmid-bearing cells are genetically more stable than their freely suspended counterparts. Several groups substantiated that this effect is an indirect result of immobilization. For instance, Barbotin et al., University of Compiégne, France, showed that when *Escherichia coli* containing the plasmid p TG201 is immobilized in K-carrageenan beads, the plasmid is twice as stable as in cells grown in continuous culture over 200 generations.

In the same laboratory, it was further shown that there was more rapid decline in plasmid number in cells at the surface of a carrageenan slab than in the interior. The process of loss of plasmids is not restricted to bacteria. Yeast containing a plasmid encoding for a *Bacillus subtilis* ß-glucanase also loses its extra-chromosomal DNA when allowed to grow in suspension. This process is much retarded when the yeast is immobilized in Ca-alginate beads, a situation which has been exploited biotechnologically. Immobilized yeast in continuous reactors secretes 20 times more ß-glucanase (an extracellular enzyme) than cells grown in batch fermentation. Again, plasmid stability was greater in the core of the beads than at the periphery.

The consensus is then that cells that have been immobilized do not grow as well as cells in free suspension. This alone causes them to retain plasmids. To this end, it is better to immobilize cells at very high densities; i.e., 10^{10}/ml, to minimize growth in the immobilization matrix (Hahn-Hägerdal, University of Lund).

In the general area of stability, it has been suggested that immobilized cells are more resistant to bacteriophage attack than free-living cells (Morin, Agricultural Institute of Canada, Ottawa).

Methods of Immobilization of Cells

Matrices. Before describing such methods, we must distinguish between passive and active immobilization. That is, between cells that become fixed in place because they colonize inert particles (glass, carbon, and celite), or microporous organic polymers (polyurethane foams) and cells that are purposely immobilized in a thermosetting- (agarose, K-carrageenan) or chemically cross-linked, organic polymer (Ca-alginate, polyacrylamide, and glutaraldehyde/protein). No fundamentally new methods were described at these meetings. However, it is instructive to review some aspects of the papers presented which describe advances in existing methods, effects of matrices themselves, and provide a few typical examples of success. We will not consider here the types of microcarrier beads used to grow anchorage-dependent mammalian cells.

Methods. For the purpose of this report, we will consider methods as standard that involve Ca-alginate, K-carrageenan, polyurethane and polyvinyl foams, agar, and polyacrylamide matrices. I will not describe the many papers that reported these techniques.

Usually, matrices of these materials are formed into beads of several millimeters in diameter by allowing the mixture to drop from a needle orifice into a curing solution; e.g., cold liquid, Ca^{2+}, or K^{+} solution. Bead size cannot be lowered much below 1 mm using current technology, and their mass production for commercial utilization is difficult. The attraction of small beads is that the cells near their center suffer less from nutrient limitation than those of larger diameter (see later remarks about the positive effects of physiologically stressing cells by immobilization in beads).

Siemann et al., Technical University of Braunschweig, FRG, described an apparatus for the reproducible mass production of beads as small as 0.25 mm. Cells and liquid matrix are fed through a spinning ring with more than 5,000 nozzles. The ring is immersed in a Ca^{2+} solution. Bead size is inversely proportional to spinning speed and not the size of the holes (nozzles) in the ring. Up to 2.8 K g / hr of Ca-alginate beads could be made with only 5 percent of them being deformed. Since bead size could so easily be controlled, it allows this to be a variable in immobilization experiments--something that is not easily obtained with beads formed by extrusion from needles. Results of cellular activity within beads was not mentioned.

Britelaar et al., Agricultural University at Wageningen, described a second method to control bead formation. Here, thermosetting gels (carrageenan, and agar) were used with three types of cells--yeast, bacteria, and plant cells. The system was based on using a membrane pump which was controlled from a sine-wave generator; i.e., the membrane resonated at the frequency produced by the generator. Results of varying the frequency were not presented, but for a given (unspecified) frequency, respiration of cells in beads in all matrices tried was considerably reduced when compared to those produced by the more usual extrusion technique. The only advantage to this resonant nozzle technique appears to be the speed of production of the beads. Although not mentioned by the authors, it should be possible to control bead size with this apparatus.

Polyacrylamide gel (PAG) is generally considered too toxic to cells to be used in immobilization experiments (acrylamide is also toxic to people!). However, some work still goes on with this polymer, probably because of the ease with which the pore size of the gel can be controlled as well as its chemical stability.

Thus, Lusta et al., U.S.S.R. Academy of Science, Moscow, reported that a series of eucaryotes and procaryotes lost viability in PAG but that resistant clones could be isolated. On the other hand, Balakrishnan, Jawaharlal Nehru University, Jabalpur, India, found that *Bacillus thermoalkanophilus* immobilized in PAG produced satisfactory amounts of endoglucanase but a little less total enzyme than free cells over the period of the experiment. However, the immobilized cells continued to produce the enzyme until the end of the experiment (10 days), whereas the freely suspended cells stopped after 6 days. If the experiment had continued for a longer time, the immobilized cells would have outstripped the suspended cells in enzyme production. These results emphasize the need to consider all matrices when choosing to immobilize cells and not to dismiss any merely because they have been found to be toxic in other systems.

Working with the ergot fungus *Claviceps purpurea*, Kren, Czechoslovak Academy of Sciences, Prague, overcame adverse effects of PAG, chitosan, and K-carrageenan as immobilizing matrices. He used a low-temperature carrageenan and modified its gel strength with locust bean gum to immobilize fungal hyphae to produce alkaloids. A further innovative suggestion by this worker was using immobilized catalase in the presence of low concentration of hydrogen peroxide in the medium. Oxygen would be generated *in situ*, thus preventing the interior of the bead from becoming O_2-depleted because of mass transfer limitations. In my opinion this procedure would have to be carefully regulated or the toxic effects of H_2O_2 would easily overcome any benefits gained. The inclusion in beads of microalgae as sources of photosynthetically derived oxygen has been suggested, but then light must be provided to the reactor interior. This is not a trivial task in a large industrial fermenter.

Problem solving was also the approach taken in a paper by Kawakami et al., Kyushu University, Japan. These workers wished to make the conversion of 1-octene to 1,2 epoxyoctane a continuous process. The approach they chose was to immobilize the biocatalyst, *Nocardia coralline*. There is often a mass transport limitation of microbial activity in matrices used to immobilize cells. In this case, the situation is compounded because the substrate for the bioconversion, 1-octene is barely soluble in the aqueous phase. Accordingly, Kawakami and coworkers immobilized Nocardia in a two-phase matrix containing a hydrophilic and a hydrophobic component. To achieve this, they used polydimethylsiloxane and Ca-alginate as well as including an aqueous component that consisted of the normal growth medium (30 percent by volume). Strictly speaking, these are three-phase beads; i.e., hydrophilic structural phase, hydrophobic structural phase, and an aqueous phase. The immobilized catalyst thus formed could be operated as a continuous multiphase bioreactor with the bathing fluid consisting of n-hexadecane. This paper is interesting to scientists involved in operating biocatalysts in nonaqueous environments since it overcomes one of the problems inherent in this type of work; i.e., the instability of the catalyst in nonpolar solvents.

Kniebusch et al., Technical University of Hamburg, FRG, also used an unusual matrix in their bioremediation work. Here, they achieved catalysis of the chemical reactions with cells adhered to the surface in addition to those inside the support, rather than the more usual method of active immobilization in a gel. They used a gas permeable

polyetherimide macroporous membrane to separate the gas and liquid streams in a flow bioreactor. Colonization of the membrane pits by bacteria exposed them on one side therefore, to oxygen and on the other to the aqueous milieu containing compounds to be broken down or bioremediated. The membrane has the oxygen permeability advantage of silicones, but is much more easily colonized by bacteria. The aim of these workers is to design small instream bioreactors to treat pollutant streams at source with highly specific biocatalysts. Such bioreactors could possibly be designed for shipboard use, especially for the treatment of oil-laden bilgewater.

Early attempts to immobilize cells often used proteinaceous materials denatured and cross-linked with small aldehydes such as formaldehyde or glutaraldehyde--compounds that are universally toxic. Alterlis et al., University of Naples, Italy, overcame this aldehyde toxicity by using the nontoxic oxidized carbohydrate starch as a cross-linking agent for gelatine. Cells could be recovered from the matrix by digesting with trypsin.

At these meetings, there was only one paper presented on a new immobilization support (rather than matrix). Dempsey described using coke as a porous, nontoxic support for immobilizing yeast in a continuous fluidized bed reactor. (Coke is the end-product of coal distillation in the production of coal-gas and tar.) Advantages to using coke over other equally cheap alternatives (sand and granite chips) are its comparatively low density and its highly porous nature. The density aids in the flotation of the colonized particles and the porosity in the protection of the adherent yeast cells. Dempsey has also used the same system to immobilize sugar beet cells. Coke seems to be a promising nontoxic substratum for immobilized biocatalyst fluidized bed reactors.

Matrix Effects. Throughout the conferences, quite naturally the theme of many papers was the inherent advantages of immobilized cells over cells in suspension for bioconversion. Although often no real reasons were advanced as to why the performance of immobilized cells should be physiologically different. Three papers addressed this question in particular.

Brits et al., Instituto Superior Técnico, Lisbon, Portugal, showed that freely suspended cells of yeast-producing ethanol and *Pseudomonas aeruginosa*-producing alginates were as active as immobilized cells if aqueous extracts of the matrix compound K-carrageenan were added to the medium. Although they had not yet identified the active fraction(s), it was suggested that Ca^{++}, Mg^{++}, or Zn^{++} were strong candidates.

Similarly, Thomasset et al., University of Compiègne, showed that the gelling ions Ca^{++} and K^{+} increased the alkaloid production of freely suspended *Coffea* and *Datura* plant cells. Further, they suggested that the immobilization process provided better cell/cell contact and allowed the establishment of the cell-generated chemical gradients that were involved in promoting secondary plant metabolism.

Although not strictly a matrix-effect, the paper by Schneider et al., National Research Council of Canada, is highly relevant to anyone working on biofilms. This group investigated the production of extracellular polymeric substances (EPS or slime) by *Pseudomonas aeruginosa* growing on surface of ultrafiltration membranes placed on nutrient agar. If the U/F membrane used allowed the diffusion of compounds of MW30-50,000 or higher, no slime was formed; i.e., EPS formation required the presence of some material of this size produced by the cell. The workers suggest that such molecules may be a component of the slime or a signal required for slime production. In this context, I suggest that this material may act as a transducer indicating that the bacterial cell is on a surface and may be therefore, an example of surface-induced change in metabolism.

Reactor Types for Immobilized Cells

Considering the number of papers presented (120) at these meetings, few of the reactor types described could be considered innovative. This may have been because most of the authors were microbiologists rather than biochemical engineers. In fact, most studies seem to have been carried out using the simplest possible equipment; i.e., a packed bed or stirred reactor, rather than other, perhaps more appropriate, systems. Few papers mentioned the reason for the choice of equipment, yet this is possibly one of the most significant variables in a process because reactor design has profound effects on mass transfer in discontinuous systems. Exceptions to these generalizations were seen in the papers of Wilderer, Technical University, Hamburg, FRG, and Dempsey. Dempsey described the reasons for using yeast immobilized in the matrices of coke particles in a fluidized bed reactor. Notable in this paper were the large flow rates able to be used, the rate of product formation (ethanol), and high glucose consumption efficiencies at the high flow rates used. Wilderer's approach was quite different. His group is designing small *in situ* bioreactors for use in treating industrial effluent streams at source. In effect, these workers have produced a large hollow fiber reactor where there is only one growth tube. The tube is a semipermeable membrane that allows gas transfer but not liquid movement. Although not strictly relevant to this report, the approach this group has taken to bioremediation of effluent streams is worth mentioning. They maintain that the practice of trying to remediate the bulked discharge from many sources is far harder than removing toxic materials from the waste of a series of single-product effluent streams. Thus, their emphasis is on small, instream, fixed-biofilm treatment devices. (The matrix used here is described more fully in the section entitled Methods of Immobilization of Cells.)

Two studies were presented where choice of reactor was central to the presentation. Looby et al., Center for Applied Microbiology and Research, Porton Down, England, found that yields of Chinese hamster ovary (CHO)

cells were higher in fixed-bed than fluidized-bed reactors. The reason for this, they speculate is particle abrasion leading to a lower cell density in the fluidized-bed reactor. Hybridoma mouse cell yield in a fixed-bed culture system was higher than in systems where the cells were freely suspended. Antibody concentration per volume of medium was also increased. Moreover, antibody production continued for 100 hours longer in the fixed-bed system than the suspended-cell system.

Baron and Van Capellen's, Free University, Brussels, paper described a new type of bioreactor that allows good mixing at the level of the individual cell-containing bead, but with very low shear at the bead surface. The reactor, which depends on the principle of Couette-Taylor flow, consists of two horizontal concentric cylinders with the inner one rotating so the annular space between them that contains the medium is mixed. The horizontal flow of the medium and the mixing caused by the rotation of the internal cylinder sets up local eddies. These eddies promote mixing. Beads containing immobilized cells are therefore subject to mixing while entrained in the eddy. This system is not similar to the annular reactor described by Siebel and Characklis, Montana State University, Bozeman, because here the width of the annulus is 10 cm. In the reactor described by Siebel and Characklis, it is less than 1 mm.

Products from Immobilized Cells

Two kinds of products can be obtained as a result of microbial activity--growth-associated primary metabolites and products of secondary metabolism. The product desired has considerable influence on the cellular immobilization method, the type of reactor chosen, as well as any process variables. To list the many products mentioned in these meetings is beyond the scope of this report. However, some general rules are emerging. If a growth-associated product is desired, it is undesirable to use cells immobilized in beads. Cellular growth leads eventually to the destruction of the bead. A system that is better for this type of product is one where cells are attached to a surface of a carrier such as glass or a plastic foam. Secondary metabolite production is sometimes favored by immobilization because in some cells, crowding along with restricted diffusion promotes this type of metabolism.

Bringi and Shuler, Cornell University, provided a striking example. In this paper, they reported that cells of tobacco, when immobilized in Ca-alginate beads, underwent cellular differentiation to produce tracheary elements. Differentiation in plant cells is considered to be a visible example of secondary metabolism. The greatest degree of differentiation took place in the center of the bead. This implies that the signal to differentiate could be related to the relatively lower concentrations of nutrients such as phosphate or O_2 in the center of the bead. Differentiation is not seen in cultures of suspended plant cells except when aggregates form.

A paper from Syldatk's laboratory, Technical University of Braunschweig, FRG, was unusual on several accounts. These workers and cells of *Chlorella* or *Synechococcus* immobilized in alginate to reduce acetyldimethylphenylsilane to hydroxyethyl dimethylphenylsilane enantiometrically. Both free and immobilized cells produced the chiral alcohol in high yield, but the immobilized cells remained active over 14 cycles without significant loss of activity. However, the free cells lost activity after one cycle, and had no activity after seven such cycles. No other paper surveyed potential matrices to the extent this one did or used microalgae in enantiometric transformations. Further, the use of immobilized biocatalysts to transform silicoorganic materials was unique.

Physiology of Immobilized Cells

Is the Physiology of Immobilized Cells Different from that of Freely-Suspended Cells? Before examining the evidence, it is pertinent to consider if there are reasons why immobilized cells and their freely living counterparts could be physiologically distinct.

Karel, University of California, Berkeley, put this quite succinctly! Do cells know they are immobilized? The most obvious difference between the environments in which these groups of cells find themselves is the degree to which free diffusion can take place. Cells entrapped in a matrix or even in a natural biofilm, are subject to diffusion limitation; i.e., the transfer of substrates to and metabolites from them is reduced because the diffusion pathways are lengthened. Free diffusion for small molecules in the gel matrices themselves is only a little slowed from that in water. Nevertheless, substrate limitation of O_2 will lead to a situation where anaerobic or at least microaerophilic conditions will exist inside a bead containing aerobic organisms. Quite obviously, this will lead to either death of the cells in the center of a bead or, if the organism is facultatively anaerobic (e.g., yeast), anaerobic metabolism. Similarly, lack of combined nitrogen without carbon limitation will lead to conditions where the composition of the immobilized cell will change towards the synthesis carbohydrate or neutral lipid/triglyercide; e.g., ß-polyhydroxybutyrate.

Of course, given the same nutritional stresses, well-mixed, free-living cells will react similarly. The exaggerated physiological response of immobilized cells therefore, may be more a product of geometry rather a physiological change per se. More specific responses of restricted diffusion are possible, however, especially where metabolic activity is controlled by feedback regulation by extracellular products. This behavior can be considered as a response to crowding of the population which in turn makes cell-cell interaction more likely. Such responses are more common among eukaryotes than procaryotes.

Evidence For and Against the Question. One of the problems in assembling evidence of this kind is that the

techniques used to decide whether various populations of cells are different physiologically from others are rarely the same from paper to paper. For instance, measurements on suspended cells are made usually when they are in the logarithmic phase, yet immobilized cells are in what is often called a pseudo-resting state; i.e., they are not growing logarithmically. In the two symposia, I counted the papers and posters where a definite decision was made; i.e., whether cells were more active, less active, or there were no physiological change detectable. Ten authors reported greater activity and five reported no change. There were no reports of cells that were less active on immobilization. In judging this result, one must bear in mind the bias of the authors. In general, they were at the meeting to promote immobilization of cells for biotechnological purposes! Below are some of the papers exemplifying perceived differences between entrapped and free cells.

In free-living cells of the cyanobacterial genus *Anabaena*, the interheterocystous distance along the filament measured in numbers of vegetative cells is constant. When filaments of *Anabaena azollae* are immobilized in polyurethane foam, heterocysts are found more frequently. Furthermore, as one might expect, nitrogen fixation on a chlorophyll basis is also increased to the extent that NH_4+ is secreted from the foam-entrapped cells. Mucilage secretion also appears to be involved in this physiological response reported by Browers et al., University of Liege, Belgium.

Bisping et al., University of Münster, showed that cells of *Pichia* (yeast) in alginate beads behaved as though they were osmotically stressed; i.e., they converted a large fraction of the growth substrate glucose to glycerol. Moreover, they underwent a morphological change from ovoid cells to a pseudomycelium. Papers on mammalian cells were not common at these two conferences, but one described a phenomenon of great practical importance and very relevant under the present heading. The CHO cells adapted to growth in serum-free medium when immobilized in collagen microspheres, whereas free-living cells did not (Venkat, The Heinz Company, Pittsburgh). This has considerable impact for animal cell biotechnology. No biochemical or physiological reasons were advanced to explain this ability of CHO cells to adapt to the removal of serum from the medium. However, it was speculated that it was because of differences in microenvironment.

Similarly, Bunch et al., University of Kent, England, showed that Streptococcus faecalis undergoes the same aerobic/anaerobic change in metabolic patterns when immobilized that it does when O_2 is withheld. Karel had made this point earlier more generally; i.e., immobilized cells that do not suffer mass transfer limitation will not behave differently from suspended cells. Evidence in favor of this explanation came from Smith et al., Agricultural University at Wageningen. When they immobilized cells of *Mycobacterium* at very low cell densities in alginate, no differences were observed between these and free cells. Reduced pyridine nucleotide generation and utilization was measured with 1,2-propene as substrate, as well as the energy status of the cells.

Again, Kuenen, Delft University, the Netherlands, using O_2-microelectrodes, showed that immobilized cells (agarose) of the mixtroph *Thiosphaera pantropha* were similar to chemostat-grown cells with respect to their growth kinetic parameters (i.e., μ_{max}, q_{O2} and $K_{acetate}$). In most cases therefore, immobilization or entrapment of cells causes a stress response, a phenomenon quite familiar to the microbial biochemist.

Stouthamer, Free University of Amsterdam, the Netherlands, made this the subject of a biochemically detailed talk--the only one in the two symposia. Stouthamer began his talk with a discussion of the rationale for the use of chemostats in studying slow growing cells. Maintenance energy is one of the parameters used in dealing with the stoichimetry of substrate utilization in a chemostat. This energy is derived from substrate metabolism that does not give rise to growth, but merely maintains the physiological status of the cell. The concept has been well accepted for the last 25 years. Whether the amount of substrate used for maintenance is independent of growth rate remains controversial. This is largely because it is extremely difficult to grow cells at very low growth rates. Where estimates of maintenance energy at low growth have been made, they are often extrapolations to zero growth rate of curves prepared by plotting growth rate (x-axis) versus substrate concentration (y). The intercept on the y-axis reflects the maintenance energy, provided the equation relating these parameters is linear. Stouthamer explained that it is not a linear relationship and the reason for excluding classical chemostat experiments from this type of work.

First, the composition of slow-growing cells is different from that of fast-growing cells. The former contain more protein, but this protein is turning over faster. There is also less efficient use of messenger RNA (m RNA) and less RNA synthesis in general. Stouthamer maintains that cells growing very slowly are really starving and therefore exhibit *alarmone* response. *Alarmone*, or "stringent response" is triggered by amino acid or energy limitation and involves guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp). Protein turnover in bacteria is proportional to the ppGpp content of the cells. Thus, slowly or barely growing cells; i.e., the types found frequently in immobilized cell reactors, are not merely biochemically less active forms of fast growing cells, but are very different indeed. Stouthamer went as far as to say that Pirt's growth equations do not apply at low growth rates. The maintenance coefficient is not a constant parameter, but one that decreases at lower growth rates. Cells relatively rich in ppGpp have lower glucose uptake rates, but synthesize more glycogen than normal cells.

Stouthamer's paper provided a fresh approach to examining the physiological changes that may occur when cells are immobilized. He proposed using a fed-batch reactor with a medium recycle loop to study this phenomenon, rather than a classical chemostat. Supporting the idea that cells immobilized in matrices exhibit a "stringent response" is suggested by the loss in activity of the biocatalyst under nongrowing conditions and its prevention by the occasional addition of low levels of growth substrates.

Noninvasive Techniques to Study Immobilized Cells. At Warwick University, there was little mention of the means by which one can study immobilized cells, *in situ*. At the Agricultural University at Wageningen, this extremely important topic was covered well. However, older methods of studying fixed cells suffered from our inability to sample for analysis. The three-dimensional structure of the film was destroyed by these methods and subsequent physiological measurements were thus invalid. Consequently, noninvasive techniques are needed. This is not to say, however, that a technique not dependent on a homogenization step is noninvasive.

Take the case of the microelectrode to measure oxygen or other chemical profiles. The electrodes, although small at the tip (1-2 μm), are still large enough to disrupt a redox gradient in a thin film. Therefore, it is important to use these electrodes with care. The redox profile measured while moving gradually in towards the substratum and through the film should be duplicated when the electrode is moved in the opposite direction (the electrode withdrawal profile). The thinner the biofilm, the more important this is.

DeBeer et al., University of Amsterdam, described how two new electrodes could be used in conjunction with a classical Clark-type-O_2-electrode to measure all the major reactants and products in natural aggregates of nitrifying bacteria; i.e., gradients of NH_4+, NO_31, and O_2. From their results, they concluded that biofilms of more than 100 μm were inefficient at oxidizing ammonia. These new types of electrodes will be useful in microbial ecology but are not sufficiently selective to use in marine systems.

The work of the Delft group (G. Kuenen et al.) on the use of O_2 microsensors has been mentioned earlier (see section entitled Physiology of Immobilized Cells).

Most of the other techniques mentioned at these symposia were physical and relied on some type of spectroscopy. In these techniques, the only invasion of the biological films is by the electromagnetic radiation used for the spectroscopy; but, in almost every case the fixed cells must be situated in the optical system of the spectrometer. This means that analysis of films in nature is not possible unless they are formed in a device such as a nuclear magnetic resonance (NMR) cuvette (for NMR spectroscopy) or on a germanium prism (for Fourier transform infra-red spectroscopy). Both of these techniques, as well as others, were covered at the Wageningen meeting.

Clark, University of California, Berkeley, reviewed most of the methods currently in use in immobilized cell investigations. From his presentation and those of Lohmeier-Vogel et al., Calgary, Canada, and Angela-Taipa, Centro de Technologica, Lisbon, Portugal, it was evident that NMR holds the most promise for future studies of the biochemistry of immobilized cells.

Further, the advent of NMR-imaging holds even greater promise. Imaging depends on proton resonating nuclei rather then those of the more usual ^{31}P or ^{13}C. Potentially, imaging NMR could be combined with the spectroscopic version so it is possible to assign specific biochemical reactions to particular cells or horizons in a biofilm. There appears to be far less potential for Fourier transform infrared spectroscopy (FTIR) since it is not selective at the level of the small molecule. The FTIR has some use as a monitoring system for instream biofilm studies, for instance. As an example, Clark cited the increase in the amide-stretching signals gathered using the attenuated internal reflectance technique that took place during the time that a culture of CHO cells grew to confluence on the surface of a germanium prism.

Clark also reviewed electron spin resonance spectroscopy. He gave no examples of its use with immobilized cells, nor did anyone else. I do not see this technique as being very useful to study cells in a matrix since it is dependent on the presence of spin-probes that must diffuse uniformly through the immobilization matrix for results to be valid.

An as yet unproved mass-spectroscopic system was described by Willaerts et al., Free University of Brussels, Belgium. The apparatus is similar to that pioneered by Bessel Kok in his work on photosynthetically produced chemical transients. An immobilized cell system is separated from the mass-spectrometry inlet by a membrane, the permeability of which specifies the type of molecule detected in the mass spectrometer. Willaerts and colleagues used a quadropole mass spectrometer and only volatile compounds were measured. At the same time, there is considerable potential for this to be developed. Those workers also mentioned a second type of reactor. In this apparatus, the membrane supporting the immobilized cells was washed continuously on its reverse side, and the washings were analyzed by an appropriate technique (electrochemistry, chromotography, mass spectrometry). I have proposed a similar reactor to study volatile acid production by microbial films involved in corrosion before, but the addition of MS-analysis would greatly improve this suggestion.

Malinski et al., Oakland University, Michigan, described a further unique method to measure cellular activity on surfaces. These workers allowed cells of baby hamster kidney (BHK) to adhere the surface of indium oxide-coated glass electrodes. They then measured the impedance and resistance of the cells. When insulin or

vanadate was added to the liquid bathing the cells, their resistance fell to a plateau of about half that of the untreated controls. Both compounds are known to modify ion-flux in mammalian cells. This technique could be used to measure the physiological status of cells in films as a function of the external concentration of some other plasma membrane perturbants of interest to the antifouling industry. There seems the possibility of using regimes of successive molecules as well as to measure biofilm penetration rates. Only mammalian cells have been investigated so far.

Most of the systems I have described are expensive. Scheper et al., University of Hannover, FRG, described a system that promises to be relatively inexpensive. Reduced pyridine nucleotides are intimately involved in metabolism and they are fluorescent at 460 nm when excited at shorter wavelengths. Thus, they form ideal reporters of cellular activity. If whole cells are excited and the emission collected using *in situ* fiber optics, the system becomes a monitor of cellular health. This idea is not new, but seems to have been used rarely in biofilm research. The idea could also be used with cells that contain the lux-genes as reporters of gene expression.

Other Methods. Some forms of microscopy can be regarded as noninvasive. However, most forms of the technique require some kind of sample preparation and these must therefore be regarded as invasive. Several workers mentioned using microscopy in their studies. I will not describe results obtained with internal reflectance microscopy, image analysis, or the various forms of electron microscopy, since these techniques are well known. Less known, especially in the field of the study of immobilized cells, is the use of the microscope as a microfluorimeter.

Ollis et al., North Carolina State University, Raleigh, measured the relative RNA and DNA contents of alginate beads at various distances from center of the bead. The lateral resolution of the analysis was about 10 μm. The results showed that specific RNA content of the beads, which is related to RNA/cell, is twice as great at the periphery of the bead as in the interior.

Again, this technique could be used with the biofilm slicing technique described by Bryers and Banks, Duke University, Durham, North Carolina, for estimates of relative cellular growth rates in fouling films. I believe this technique is sufficiently novel to bear more detailed description. The goal of this laboratory is to understand the processes controlling the population dynamics during the formation of a biofilm. As pointed out by these workers, films in nature are always of the mixed variety. Yet because of the complexity of modeling mixed films, most investigators work with monospecific films. Completely natural films are almost useless as study systems at this stage of our knowledge since we cannot yet assess quantitatively the organisms in a film.

Bryers and Banks described two types of systems. The first, a natural enrichment of acetate-oxidizing organisms, was mixed with an autotrophic enrichment of nitrifying bacteria. A second series of experiments were carried out with a biofilm containing the heterotroph *Peudomonas putida* and a *Hyphomicrobium* species. These organisms were metabolically distinct in that the *Pseudmonas* metabolized only glucose and the *Hyphomicrobium*, methanol in the mixed glucose/methanol reactor feed. The reactor was a small duct 2.5-cm wide by 0.1-cm deep and 7.6-cm long, which allowed the placement of standard microscope slides on its base. Removing a slide gave a more or less undisturbed section of the biofilm in the duct as a sample. To enumerate the organisms in a film, a sample of the biomass was removed as described above and incubated with labeled substrates ($NaH^{14}CO_3$ and ^{3}H-acetate for the autotroph/heterotroph biofilm, and $^{14}CH_3OH$ and ^{3}H-glucose for the mixed heterotrophs). After incubation, the labelled films were washed, fixed, and embedded in electron microscopy sectioning resin. Slices of the embedded film (20 μm) were placed in appropriate cocktail in a liquid scintillation counter set for double-label counting. Previous calibration experiments allowed the results to be expressed as biomass dry weight of cells per slice, thus making it possible to determine spatial variation of cells with time.

Using these techniques, Bryers and Banks showed that microscopical examination of the effluent from a reactor does not give the species composition of the film (unless the film is uniform and both species of cells slough at exactly the same rate). The system can be adapted to use immunofluorescence as the detection system rather than radioactivity. In this case, dead as well as live cells would be counted. These workers have coupled image analysis to the examination of unfixed cells. However, this technique could also be used to assess radioactivity in individual cells after auto radiography of the biofilm slices.

In this work, no account appeared to be taken of inter-species metabolite transfer or anaplerotic CO_2-fixation by heterotrophs. This could be quite significant in cells growing on 1- or 2-carbon substrates. In spite of these reservations, this technique to measure species dynamics in a mixed film is very useful and can be adapted to assess inter-species carbon transfer.

Modeling the Metabolic Activities of Immobilized Cells

The lesson to be learned from the two symposia was that so far most modeling effort have been "naive and glib" (Hamer, ETH, Zurich) or perhaps based on "fantasy" (Karel et al., Princeton University, New Jersey)! The most critical of the two is Hamer. His basic complaint rests largely with the engineering community and their slowness to appreciate the fact that linear models of biological phenomena rarely fit completely, usually because the biological process is not linear! Furthermore, just because a linear model appears to fit a process well does not mean it is predictive. He believes that the universal application of material balance equations that

assume that cells have constant composition irrespective of their growth rate is too simplistic (see earlier remarks in Physiology of Immobilized Cells).

Other problems that Hamer sees concern the general lack of appreciation of the influence of microenvironments. This is compounded by the fact that modelers usually base their equations on measurements made in the macroenvironment.

Developing this further, Hamer believes there are no true steady states since there are gradients everywhere. Although it might be argued that diffusion patterns, at least in a laminar flow situation, are uniform, it is more than likely that they are not because of the existence of microturbulence or eddies. The influence of such eddies on mass transfer to cells from media undergoing laminar flood is hard to predict.

An example of the concerns, as expressed by Hamer, is demonstrated in a paper given at the same symposium. Siebel and Characklis, Montana State University, modeled a mixed biofilm of *Klebsiella pneumonia* and *P.aeruginosa*. The organisms are treated as aerobic heterotrophs, which under conditions of oxygen excess, they are. However, in conditions other than this, Klebsiella is facultatively anaerobic. Thus, specific glucose consumption rate will not be related linearly to the oxygen consumption rate--a fact shown by their experimental data but not by the model presented.

Karel and his coauthors at Merck, Sharp, and Dohme, and Stanford University, Stanford, California, echoed some of Hamer's thoughts concerning microenvironments and their influence on cellular activity in films and matrices. Neither the other papers presented nor my previous experience in the field convinced me that the reservations expressed by Hamer and Karel were unfounded or exaggerated.

Geographic Distribution of Papers and National Emphasis

One should not predict the location of centers of research activity based on a small number of papers presented at one geographic site. In this case, there were two sites involved and 120 papers, so I feel comfortable in doing so.

The major centers of activity in immobilized cell research are in the Netherlands, the U.K., and the FRG. This distribution agrees with my perception of the relative scale of biotechnological research in Europe. The leaders in Europe are followed closely by France, Belgium, and Spain. Seventeen other countries were represented at these conferences. Notable centers of research in Europe are the University College of London, University of Campiégne, France, the Institute of Microbiology, Bulgarian Academy of Science, Sofia, Bulgaria, and Agricultural University at Wageningen, the Netherlands. The expertise in immobilized cell research is not concentrated in a particular center in Germany, but the Technical University of Braunschweig has an active group. The use of particular techniques is randomly distributed, but there is a greater emphasis on sophisticated, and thus expensive, physical instrumentation in the U.S. than in Europe.

Research Needs

The techniques used to immobilize cells for biotechnological purposes are not new, nor are the matrices that are used. As this sphere of activity progresses and begins to encompass organisms from other areas of biotechnology, better means of immobilizing cells will be needed. For instance, no papers at either meeting concerned the biotechnological uses of thermophilic microorganisms. Yet there is a large research effort aimed at using organisms at elevated temperatures. Few of the matrices used now are useful at higher temperatures, and those that would be stable may well be toxic. There is a need to find a replacement for Ca-alginate. Calcium is a regulatory ion, especially in eukaryotes, and to immobilize a cell in a high Ca environment seems counter productive to achieving catalytic stability. Greater stability of the biocatalytic activity is a need even at mesophilic temperatures. Organisms immobilized in a nongrowing state lose activity after a few cycles in the reactor. They can be reactivated by allowing a short period of growth to take place. To understand how this phenomenon happens is important. What is the mechanism of inactivation and reactivation of immobilized cells? Possible mutants could be exploited that can metabolize at some basic low maintenance level, but cannot divide. They should be catalytively more stable than wild-type cells.

Those interested in the activities of natural biofilms may be able to exploit immobilized cell technology to form model-defined films. However, since natural films always consist of more than one organism, it will be important to understand how films of mixed species can be made and to what extent organism-organism interaction takes places. Such mixed films could be used for multi-step reactions, or one organism could be involved in generating the conditions, for the other to operate; e.g., E_h.

Research with organisms in films will be facilitated if we have a greater battery of noninvasive techniques to exploit. Nuclear magnetic resonance spectroscopy seems to be the method of choice, but this is expensive and requires a special reactor to be constructed that fits the measurement well of the instrument. The current microelectrodes are still rather large compared to the size of a film of cells only a few cells thick. Smaller electrodes would be an advantage as well as electrodes with other chemical sensitivities. Better, non-invasive techniques will enhance greatly our knowledge about the interactions between cells in films and matrices.

Greater knowledge of the physiology of entrapped cells will allow more accurate modeling scenarios to be proposed, especially those dealing with nonlinear

responses and cellular interactions. Very few modelers have tackled the phenomenon of cellular interaction.

Finally, I believe that advances in all of these fields will allow the subject of microbially-enhanced corrosion to be studied in an artificial system where corrosion rates can be obtained that are similar to those in nature. A mixture of the correct organisms in a nontoxic matrix is necessary. The source of the metal could be colloidal iron particles included in the matrix. Further, if matrix containing cells and iron were mounted on a semipermeable membrane, chemical analysis of reactions within the film could be monitored by any one of several techniques mentioned earlier.

Using microorganisms in biotechnological endeavors is almost as old as civilized man. Their immobilization seems to offer us a means to control more closely their catalytic processes.